SUMMARY

1. A phospholipid-splitting enzyme of *Vibrio* El Tor has been characterized as a phospholipase B (lysolecithinase).

2. The enzyme is secreted during the late log phase of growth and no further increase in the excretion has been observed during the stationary phase.

3. The enzyme has been isolated from the culture filtrates by ammonium sulphate fractionation between 50 and 75% saturation and some of the properties of the enzyme preparation have been studied.

4. This *Vibrio* El Tor lysolecithinase has a pH optimum at 8·0, and is fairly stable towards inactivation by heat. The enzyme is inhibited by high concentrations of substrate. It is fairly insensitive to the action of metal ions but is inhibited by Zn^{2+} ions (mM) to the extent of about 40%.

The authors are indebted to Professor B. C. Guha for his kind interest, to Dr R. M. C. Dawson, Cambridge, for his gift of a sample of glycerophosphorylcholine, and to Dr K. L. Roy, Department of Applied Chemistry, Calcutta University, for his help in the preparation of ferric perchlorate.

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Biochem. J. (1962) **83**, 387

Regulation of Glucose Uptake by Muscle

6. FRUCTOSE 1,6-DIPHOSPHATASE ACTIVITY OF RAT HEART AND RAT DIAPHRAGM*

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(*Received* 7 *November* 1961)

Newsholme & Randle (1961) presented evidence that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate in perfused rat heart is increased by anoxia and decreased by starvation in a specific way. Essentially similar findings with anoxia have been reported by Regen, Davis & Morgan (1961) who also observed that the overall rate of phosphorylation of fructose 6-phosphate is decreased in alloxan diabetes. The conclusion was drawn that these agents must affect either the rate of conversion of fructose 6-phosphate into the diphosphate (catalysed by phosphofructokinase) or that of the reverse reaction [catalysed by fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11)]. The present paper is concerned with the fructose 1,6-diphosphatase activity of extracts of hearts from normal and alloxan-diabetic rats and from normal rats subjected to prolonged starvation.

* Part 5: Newsholme & Randle (1961).

There is little information concerning fructose 1,6-diphosphatase in muscle. Lohman (1933) observed that inorganic phosphate was liberated when extracts of frog muscle were incubated with fructose 1,6-diphosphate but could not obtain consistent activity with extracts of rabbit skeletal muscle. Hers (1957) found that inorganic phosphate was liberated when extracts of rabbit skeletal muscle were incubated with fructose 1,6-diphosphate, and that this activity is enhanced by glyoxaline, histidine or tris. The conclusion was drawn that fructose 1,6-diphosphatase is present in muscle but it was not shown in these experiments that the liberation of phosphate from fructose 1,6-diphosphate is associated with the formation of fructose 6-phosphate. Weber & Cantero (1959) found that little inorganic phosphate is liberated when extracts of rat muscle (unspecified) were incubated with fructose 1,6-diphosphate, and concluded that the fructose 1,6-diphosphatase activity of rat muscle is less than 0·1 % of that of rat liver. In liver the presence of fructose 1,6-diphosphatase appears to have been unequivocally demonstrated. Thus liver extracts have been shown to form both phosphate and fructose 6-phosphate from fructose 1,6-diphosphate; the enzyme has been partially purified and some of its properties have been investigated; and changes in the activity of the enzyme have been reported to follow changes in the physiological state of the animal (Gomori, 1943; Hers, 1957; Pogel & McGilvery, 1952; McKrasch & McGilvery, 1956; McKrasch, Davidson & McGilvery 1956). In investigating the fructose 1,6-diphosphatase activity of heart and diaphragm muscle we have studied the formation of both inorganic phosphate and fructose 6-phosphate from the diphosphate and compared the activity of muscle extracts with those prepared from liver under essentially similar conditions.

MATERIALS AND METHODS

Rats. Hearts and diaphragms were obtained from male and female albino Wistar rats, of 200–300 g. and 100–150 g. respectively, starved for either 18 or 30 hr. Alloxan diabetes was induced by the intravenous administration of alloxan (60 mg./kg.) under ether anaesthesia. The animals were used 48 hr. later and after starvation for 18 hr., and all had blood-glucose concentrations (after starvation) greater than 300 mg./100 ml.

Chemicals and enzymes. Hexose phosphates, coenzymes and enzymes were obtained from the sources stated by Newsholme & Randle (1961). Alloxan and *p*-methylaminophenol sulphate were obtained from Eastman Kodak Co., Rochester, U.S.A.

Dialysis tubing. Visking seamless cellulose $\frac{8}{32}$in. tubing (from Hudes Merchandising Corp. Ltd., London, W. 1) was soaked overnight in 25 mM-urea and mM-EDTA, and washed thoroughly with water.

Hearts and diaphragms. These were perfused and incubated under conditions described previously (Newsholme & Randle, 1961). At the end of perfusion or incubation the muscle was either frozen immediately in acetone–solid CO_2 or chilled in 0·9 % NaCl at 0° (in which case extraction was begun within 1–2 min.).

Extracts. Frozen muscle was powdered in a percussion mortar cooled in solid CO_2. A weighed quantity of powder was extracted rapidly with a pestle and mortar with buffer solution (2 ml./g.) (either 0·25M-sucrose and 10 mM-tris, pH 7·4, or 0·25M-sucrose and 50 mM-$NaHCO_3$ gassed with air containing 5 % of CO_2). Non-frozen muscle or liver was chilled in 0·9 % NaCl at 0°, weighed, cut into small pieces with scissors and extracted in a manually operated Potter–Elvehjem homogenizer with buffer (2 ml./g.) (as above). Extracts (of either frozen or non-frozen tissue) were centrifuged to remove gross debris (at approx. 800*g* for 2 min. at 0°), and the supernatant was used for diphosphatase assay either directly or after 4 hr. of dialysis against water at 4°.

Cell fractions. Hearts were cut into small pieces with scissors and disrupted (in 0·25M-sucrose, 2 ml./g.) in a manually operated Potter–Elvehjem homogenizer (disruption was indicated by free and easy movement of the plunger and usually required 10–15 turns). A portion (10 ml.) of this homogenate was layered on 10 ml. of 0·35M-sucrose in a 50 ml. centrifuge tube and cell debris removed by centrifuging at 490*g* for 10 min. at 0°. The top layer was removed and layered on 10 ml. of 0·5M-sucrose and spun at 3250*g* for 20 min. at 0°. The pellet, consisting largely of mitochondria [identified by light microscopy; slight contamination with microsomes was shown by a low RNA content, assayed by Mr D. E. S. Truman of this Department—see Truman & Korner (1962)], was dispersed in 0·25M-sucrose in a hand-operated homogenizer (2 ml./g. of original muscle). The top layer of the supernatant was spun at 105 000*g* in a Spinco Model L preparative ultracentrifuge for 1 hr. at 0°; this pellet (dispersed in 0·25M-sucrose, 2 ml./g. of original muscle) is referred to as the microsomal fraction and this supernatant as the soluble fraction.

Fructose 1,6-diphosphatase activity. This was assayed by the formation of hexose monophosphates (glucose 6-phosphate and fructose 6-phosphate) and inorganic phosphate from fructose 1,6-diphosphate (in some assays inorganic phosphate was not estimated). Values were corrected for endogenous hexose monophosphate and inorganic phosphate by control incubations from which fructose 1,6-diphosphate was omitted. The assay mixture consisted of 0·1 ml. of liver or muscle extract or cell fractions and 0·9 ml. of buffer containing $MgCl_2$ (50 mM) and 5 μmoles of fructose 1,6-diphosphate (sodium salt). Incubation was for 20 min. at 37° and the reaction was stopped and protein precipitated by adding 0·5 ml. to 1·5 ml. of boiling water and boiling for 10 min. After cooling and removal of precipitated proteins by centrifuging, hexose monophosphates and inorganic phosphate were estimated in the supernatant. Control experiments showed that no significant hydrolysis of hexose monophosphates or fructose 1,6-diphosphate occurred during boiling; that the recovery of hexose monophosphates after addition of 7·5 μmoles of glucose 6-phosphate to the assay mixture with dialysed muscle extracts was 90–100 %; and that the utilization of fructose 1,6-diphosphate under any of the conditions employed was always less than 50 % of

that added. Assays were performed at pH 5·5, 7·4 and 9·5 by using two buffers at each pH [pH 5·5: 50 mM-acetate (sodium salt) or 0·1 M-citrate (sodium salt); pH 7·4: 50 mM-$NaHCO_3$ gassed with 5% CO_2 in air or 0·1 M-tris adjusted with HCl; pH 9·5: 50 mM-($NaHCO_3 + Na_2CO_3$) or 0·1 M-glycine adjusted with NaOH]. Since the results at a given pH with each buffer were essentially the same they have been pooled. Details of any variation in the experimental conditions are given in the Tables.

Fructose 1,6-diphosphatase activity was expressed as μmoles of product (hexose monophosphate or inorganic phosphate) released/g. of wet tissue/hr.

Hexose monophosphates. Glucose 6-phosphate and fructose 6-phosphate were assayed together by the change in extinction at 340 mμ on the addition of 5 μg. of glucose 6-phosphate dehydrogenase and 50 μg. of glucose phosphate isomerase to a mixture of 0·2 ml. of deproteinized supernatant from the diphosphatase assay and 2·8 ml. of reaction mixture (60 mM-tris; 3 mM-EDTA; 0·1 mM-NADP; pH 8·0).

Inorganic phosphate. This was assayed photometrically with 0·5 ml. of deproteinized supernatant, 5 ml. of acid molybdate (5 g. of ammonium molybdate and 25 ml. of concentrated H_2SO_4/l.) and 1 ml. of reducer (Harris & Popat, 1954) diluted to 10 ml. with water and read at 810 mμ 45 min. later.

RESULTS

When extracts of rat diaphragm muscle or of non-frozen or frozen hearts from normal rats (starved for either 18 or 30 hr.) or from alloxan-diabetic rats were incubated with fructose 1,6-diphosphate, some hydrolysis to fructose 6-phosphate and inorganic phosphate was detected (Tables 1 and 3). The activity was in all instances very low (0–5·7 μmoles/g. of muscle/hr.) and amounted to less than 5% of that of comparable extracts of rat liver. Since the high activity of liver extracts was not appreciably diminished by mixing with extracts of rat-heart muscle, the low activity of the latter is unlikely to be due to the presence of inhibitory substances. The activity of heart extracts, like that of liver extracts, was greatest at pH 9·5. When the activities of extracts from hearts of normal rats starved for 30 hr. or from alloxan-diabetic rats were compared with those of normal rats starved for only 18 hr., no marked or consistent differences were seen. There was however some indication that the activities at pH 5·5 or 7·4 might be increased by starvation or diabetes. The rates of hydrolysis of *p*-nitrophenyl phosphate and of glycerol 2-phosphate by heart-muscle extracts were greater than that of fructose 1,6-diphosphate; on the other hand liver extracts hydrolysed fructose 1,6-diphosphate at a greater rate. The hydrolysis of glycerol 2-phosphate by muscle extracts, like that of fructose 1,6-diphosphate, was most rapid at pH 9·5; the hydrolysis of *p*-nitrophenyl phosphate was most rapid at pH 5·5.

The hydrolysis of fructose 1,6-diphosphate to fructose 6-phosphate by soluble, mitochondrial and

Table 1. *Hydrolysis of fructose 1,6-diphosphate, p-nitrophenyl phosphate and glycerol 2-phosphate by extracts of rat heart, diaphragm and liver*

Hydrolysis rates are given as μmoles of product/g. wet tissue/hr. (products estimated: glucose 6-phosphate and fructose 6-phosphate with fructose 1,6-diphosphate; *p*-nitrophenol with *p*-nitrophenyl phosphate; phosphate with glycerol 2-phosphate). The number of tissue samples studied at each pH is given in parentheses.

Type of extract and substrate	Dialysis	Hearts from normal rats starved for 18 hr. pH...5·5	7·4	9·5	Hearts from normal rats starved for 30 hr. 5·5	7·4	9·5	Hearts from alloxan-diabetic rats 5·5	7·4	9·5	Livers from normal rats 5·5	7·4	9·5	Diaphragms from normal rats 5·5	7·4	9·5
Non-frozen tissue with fructose 1,6-diphosphate	–	1·7±0·3 (4)	1·6±0·1	4·7±0·8	1·4±0·2 (4)	1·4±0·2	5·7±0·3	1·6±0·2 (4)	0·8±0·4	4·1±0·4	35±4 (6)	30±5	91±4	·	·	·
	+	2·0±0·2 (4)	0·2±0·1	4·6±0·1	2·7±0·1 (4)	0·8±0·1	3·3±0·1	2·5±0·1 (4)	1·6±0·3	4·9±0·6	·	·	·	·	·	·
Frozen muscle with fructose 1,6-diphosphate	–	0·4±0·1 (4)	0·1±0·2	1·8±0·1	0 (4)	0	0·9±0·1	1·9±0·2 (4)	1·1±0·2	1·6±0·1	44±1 (3)	58±5	44±4	0·6±0·3 (3)	0·6±0·1	0·2±0·1
	+	1·5±0·2 (4)	0·4±0·1	1·0±0·8	2·4±0·3 (4)	0·8±0·1	3·1±0·1	2·2±0·2 (4)	0·5±0·1	2·5±0·1	32±3 (3)	31±5	36±1	0·7±0·1 (3)	0·2±0·1	0·0
Non-frozen tissue with *p*-nitrophenyl phosphate	–	9·0±0·9 (3)	4·5±0·3	6·3±0·3	·	·	·	·	·	·	·	·	15±1	·	·	·
	+	23±0·5 (3)	5·5±0·8	11±1·7	·	·	·	·	·	·	·	·	9±1·7	·	·	·
Non-frozen muscle with glycerol 2-phosphate	–	4·0±1	8·0±0·1	25±2	11±1	9±1	25±2·7	·	·	·	·	·	·	·	·	·

microsomal fractions of hearts from normal and diabetic rats is shown in Table 2. The activities of all three fractions, like those of extracts of heart muscle, were extremely low.

The effect of replacing Mg^{2+} ions by Ca^{2+} ions in the incubation medium on the hydrolysis of fructose 1,6-diphosphate to fructose 6-phosphate and inorganic phosphate is shown in Table 3. In liver extracts the rate of hydrolysis was reduced by about 85% when Mg^{2+} ions were replaced by Ca^{2+} ions; in heart-muscle extracts no such change was detected.

Hers (1957) reported that the formation of inorganic phosphate from fructose 1,6-diphosphate by extracts of rabbit skeletal muscle is markedly stimulated by glyoxaline, histidine or tris. We therefore investigated the effect of these substances on the formation of fructose 6-phosphate and inorganic phosphate from fructose 1,6-diphosphate by extracts of rat heart. The results are given in Table 4, and show that glyoxaline, histidine or tris increases phosphate production without, however, increasing fructose 6-phosphate production. The findings of Hers are thus confirmed but, since fructose 6-phosphate was apparently not formed when phosphate was liberated from fructose 1,6-diphosphate, it seems unlikely that fructose 1,6-diphosphatase was involved in the liberation of

Table 2. *Hydrolysis of fructose* 1,6-*diphosphate by cell fractions of rat heart*

Hydrolysis rates are given as μmoles of glucose 6-phosphate and fructose 6-phosphate formed/g. of wet tissue/hr. Numbers of experiments are given in parentheses.

Tissue	pH	Soluble fraction	Mitochondria	Microsome
Hearts from normal rats	5·5	0·0 (4)	0·1 (3)	0·0 (4)
	7·4	0·0	0·1 (3)	0·4
	9·5	0·4	0·1	0·0
Hearts from diabetic rats	5·5	0·0 (4)	0·0 (3)	0·1 (3)
	7·4	0·0	0·7	0·4
	9·5	0·0	0·0	0·5

Table 3. *Hydrolysis of fructose* 1,6-*diphosphate by extracts of rat heart and liver, separately and combined: effects of* Ca^{2+} *ions and* Mg^{2+} *ions*

Hydrolysis rates are given as μmoles of glucose 6-phosphate and fructose 6-phosphate or of inorganic phosphate formed/g. of wet tissue/hr. Numbers of experiments are given in parentheses.

pH	Metal ion (50 mM)	Heart (hexose 6-phosphates)	Heart (inorganic phosphate)	Liver (hexose 6-phosphates)	Liver (inorganic phosphate)	Heart and liver combined (hexose 6-phosphates)
5·5	Mg^{2+}	0·9 (6)	0·9 (6)	39 (4)	.	33 (4)
	Ca^{2+}	0·8 (2)	0·0 (2)	.	.	.
7·4	Mg^{2+}	0·3 (8)	0·7 (8)	60 (4)	.	58 (4)
	Ca^{2+}	0·5 (4)	1·5 (4)	.	.	.
9·5	Mg^{2+}	1·2 (8)	2·6 (8)	51 (4)	.	52 (4)
	Ca^{2+}	0·6 (6)	6·7 (6)	.	.	.
	Mg^{2+}	.	.	25 (4)	29 (6)	.
	Ca^{2+}	.	.	0 (4)	5 (4)	.

Table 4. *Formation of inorganic phosphate and of glucose* 6-*phosphate and fructose* 6-*phosphate from fructose* 1,6-*diphosphate by extracts of rat heart*

Rates are given as μmoles of glucose 6-phosphate and fructose 6-phosphate or of inorganic phosphate/g. of wet tissue/hr. Numbers of experiments are given in parentheses. Extracts were prepared from hearts of normal rats and dialysed (see Materials and Methods section). Boiled extracts were heated at 100° for 15 min.

			Heart extract		Boiled heart extract	
Buffer	pH	Aldolase (0·2 mg./ml.)	Hexose 6-phosphates	Inorganic phosphate	Hexose 6-phosphates	Inorganic phosphate
0·15M-Glyoxaline	6·1	−	0·6 (6)	36 (6)	1·6 (3)	4 (3)
		+	.	.	1·1 (3)	49 (3)
70 mM-Histidine	7·1	−	0·6 (6)	34 (6)	.	.
0·15M-Tris	9·0	−	1·2 (4)	45 (4)	0·5 (3)	6 (3)
		+	.	.	0·5 (3)	84 (3)

phosphate. Another possibility was that the phosphate is derived from triose phosphates (formed by the action of aldolase in muscle extracts on fructose 1,6-diphosphate). Heart extracts were therefore heated to 100° for 15 min. and then incubated with and without the addition of crystalline aldolase. In the absence of added aldolase the production of fructose 6-phosphate by the heated extracts was essentially unchanged whereas the production of inorganic phosphate was substantially reduced. When aldolase was added the production of inorganic phosphate was very greatly increased whereas that of fructose 6-phosphate remained at the previous low rate (Table 4). We conclude therefore that the high rate of phosphate production from fructose 1,6-diphosphate by muscle extracts in the presence of glyoxaline, histidine or tris is due not to fructose 1,6-diphosphatase but to aldolase. We suggest that the phosphate is derived from triose phosphates by hydrolysis.

DISCUSSION

The present studies were prompted by the observation that the overall rate of phosphorylation of fructose 6-phosphate to the diphosphate in perfused rat heart is increased by anoxia and diminished by starvation and diabetes (Newsholme & Randle, 1961; Regen *et al.* 1961). Further, it has also been found that the overall rate of phosphorylation of fructose 6-phosphate is increased by salicylate and depressed by the oxidation of ketone bodies, fatty acids and pyruvate (Newsholme, Randle & Manchester, 1962). These changes could result from alterations in the activity of either the phosphofructokinase step or the fructose 1,6-diphosphatase step. The present studies appear to show that if fructose 1,6-diphosphatase is present at all in rat-heart muscle its activity is extremely low and does not affect significantly the overall phosphorylation of fructose 6-phosphate. Thus the highest rate of hydrolysis of fructose 1,6-diphosphate by rat-heart extracts was 5·7 μmoles/g./hr. This is very small by comparison with the phosphofructokinase activity of heart-muscle extracts (about 45 μmoles of fructose 1,6-diphosphate formed/g./hr.). The rate of phosphorylation of fructose 6-phosphate in the perfused rat heart may be calculated approximately by subtracting from the glucose phosphorylated that portion which is converted into glycogen. This yields a value of approximately 44 μmoles/g./hr. for hearts perfused aerobically with insulin (0·1 unit/ml.) and glucose (1 mg./ml.). This is increased, for example, with anoxia, to at least 110 μmoles/g./hr., and reduced by ketone bodies to 27 μmoles/g./hr. (cf. Williamson & Krebs, 1961). It is difficult to see how changes in the very low fructose 1,6-diphosphatase activity of heart muscle which we have observed could account for the very much greater changes in the overall rate of phosphorylation of fructose 6-phosphate. We conclude therefore that the effects of anoxia, salicylate, starvation, diabetes and the oxidation of ketone bodies, fatty acids and pyruvate on the overall rate of phosphorylation of fructose 6-phosphate in the perfused rat heart are brought about by changes in the rate of the phosphofructokinase step.

It is difficult to conclude with certainty that fructose 1,6-diphosphatase is completely lacking in rat-heart and diaphragm muscle. The activities found are extremely low and not much greater than that of extracts which had been heated at 100° for 15 min. Moreover, the rate of hydrolysis of fructose 1,6-diphosphate was less than that of *p*-nitrophenyl phosphate or glycerol 2-phosphate and was unaffected by replacing Mg^{2+} ions with Ca^{2+} ions, and some phosphatase other than fructose 1,6-diphosphatase may have been responsible for the activity. Nevertheless, the possibility remains that the enzyme is present at extremely low concentration. Even if this is so, however, it seems unlikely that the concentration is sufficient to enable significant reversal of glycolysis to take place in these tissues.

SUMMARY

1. The fructose 1,6-diphosphatase activity of rat heart and diaphragm muscle has been investigated by measurement of the rate of formation of fructose 6-phosphate and inorganic phosphate from fructose 1,6-diphosphate.

2. The fructose 1,6-diphosphatase activity of extracts of diaphragm or of hearts from normal rats (starved for 18 or 30 hr.) or from alloxan-diabetic rats was extremely low (0–5·7 μmoles of product formed/g. of wet muscle/hr.) and not more than 5% of that of comparable extracts of rat liver. It is also extremely low by comparison with the phosphofructokinase activity of extracts of rat heart (45 μmoles/g./hr.).

3. It is concluded that there is very little, if any, fructose 1,6-diphosphatase in rat heart or diaphragm muscle; that changes in the overall rate of phosphorylation of fructose 6-phosphate in these tissues must be brought about by alterations in the activity of the phosphofructokinase step; and that the activity of fructose 1,6-diphosphatase is too low to permit significant reversal of glycolysis to take place in these tissues.

We wish to thank the British Diabetic Association and the Medical Research Council for contributing to the cost of these investigations; and Mrs W. Thorne for skilled technical assistance. One of us (E.A.N.) holds a D.S.I.R. studentship. It is a pleasure to thank Professor F. G. Young for his interest and encouragement.

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Biochem. J. (1962) **83**, 392

Oxidation of Short-Chain Fatty Acids (C_1–C_8) by Ram, Bull, Dog and Fowl Spermatozoa

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(*Received* 12 *June* 1961)

Mammalian spermatozoa can utilize a glycolysable carbohydrate, such as the fructose usually present in seminal plasma, under both aerobic and anaerobic conditions, but in its absence motility is maintained by the oxidation of intracellular reserves (Mann, 1954). Hartree & Mann (1959) demonstrated that fatty acids are liberated when washed ram spermatozoa are incubated under aerobic conditions, and the low R.Q. (0·71) suggests that fatty acids participate in aerobic endogenous metabolism. Information on the oxidation of fatty acids by spermatozoa, however, is scanty and confusing. Lardy & Phillips (1944; 1945) found that acetate stimulated the oxygen uptake of bull epididymal spermatozoa, but with washed ejaculated spermatozoa the effect was consistently obtained only in the presence of 2,4-dinitrophenol. Similar results were obtained by Humphrey & Mann (1948) with washed ejaculated ram spermatozoa with acetate, propionate and butyrate as substrates. Flipse (1960) found that acetate increased the oxygen uptake of washed bull spermatozoa appreciably in the absence of 2,4-dinitrophenol, but no stimulation was obtained with butyrate and higher fatty acids. Terner (1960) showed that human spermatozoa can oxidize [1-^{14}C]acetate.

In the present study the oxidation of short-chain fatty acids (C_1–C_8) by ram, bull, fowl and dog spermatozoa has been investigated with ^{14}C-labelled substrates. The interrelation of acetate and propionate oxidation by ram and dog spermatozoa has also been investigated.

MATERIALS AND METHODS

Preparation of suspensions of washed spermatozoa. Semen was collected by the techniques described by Scott, White & Annison (1961). Ram and bull semen was placed in a water bath at 37° immediately after collection and slowly cooled to room temperature to avoid cold shock. Ram and bull spermatozoa were washed twice with calcium-free Krebs–Ringer phosphate or bicarbonate buffer (Krebs, 1933) by the procedure of White (1953); the final concentration in the suspensions was 4×10^8–8×10^8 spermatozoa/ml. The diluent for fowl semen was a modified Krebs–Ringer phosphate with no added fructose (Wales & White, 1960). Fowl semen was diluted 1:4 and centrifuged at 300*g* for 10 min.; the supernatant was removed and the spermatozoa were resuspended at a concentration of about 8×10^8/ml. The sperm-rich fraction of dog semen was diluted 1:4 with calcium-free Krebs–Ringer phosphate and the spermatozoa were washed by the procedure outlined for fowl spermatozoa; the final cell concentration of the suspension was 10^8/ml. The spermatozoa were resuspended in the diluent used for washing and spermatozoal counts were made with a haemocytometer (White, 1953).

Incubation of spermatozoa with ^{14}C-labelled fatty acids. Suspensions of spermatozoa (2 ml.) were incubated at 37° with the sodium salts of the fatty acids (1 ml.) in Warburg flasks containing CO_2-free 20% (w/v) NaOH (0·2 ml.) in the centre well. Oxygen uptakes were measured with air as gas phase, and a shaking rate of 120 strokes/min., over a period of 2–3 hr. At the end of the incubation the contents